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Site-specific cleavage of natural mRNA sequences by newly designed hairpin catalytic RNAs

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ABSTRACT

The negative strand of tobacco ringspot virus satellite RNA is a self-cleaving RNA. Its catalytic domain and substrate domain have been identified, and the catalytic domain has been named hairpin catalytic RNA. Here we report the construction of a plasmid containing a modified hairpin catalytic RNA sequence that can be transcribed *in vitro*. Because this plasmid has two specific restriction enzyme recognition sites at both ends of the substrate binding site in the catalytic RNA sequence, it is possible to construct new plasmids by substituting different sequences in the substrate binding site. Using this plasmid, synthetic DNA, and *in vitro* transcription, we obtained three ribozymes designed to cleave *Escherichia coli* prolipoprotein signal peptidase (*lsp*) mRNA at specific sites. All three ribozymes cleaved the *lsp* mRNA sequence *in vitro* at the specific sites, and two of them cleaved it efficiently. Kinetic analyses showed that one had a higher k_{cat}/K_m value than that of the well-known hammerhead ribozyme. Problems associated with attaining the goal of expressing these ribozymes *in vivo* also are discussed.

INTRODUCTION

A number of small plant viral satellite, virusoid, and viroid RNAs contain self-cleaving sequences (1–6). From the conserved primary and secondary structures of the self-cleaving domain, many of them belong to the category of the hammerhead ribozymes (4; compiled by G. Bruening, ref. 6). The self-cleaving domain of hammerhead ribozymes can be separated into catalytic and substrate parts, and the catalytic part interacts with the substrate as a true enzyme (7). General rules have been deduced for designing new hammerhead ribozymes with high sequence-specific endoribonuclease activity (8–10), and several such ribozymes have been found to correctly cleave various RNA sequences *in vitro* (8–17).

The negative strand of satellite RNA of the tobacco ringspot virus [(−)sTRSV] is also a self-cleaving RNA (3, 4). (−)sTRSV RNA is autocatalytically cleaved at a specific site in the presence of Mg^{++} to generate a 2′,3′ cyclic phosphate end and a 5′ hydroxyl end similar to the hammerhead ribozymes. However, no structure in the catalytic domain resembles that of the hammerhead ribozyme (3, 4). A catalytic domain consisting of 50 nucleotides and a substrate domain of the 14 nucleotides have been identified in the (−)sTRSV RNA sequence (18–22). Since the RNA forms a hairpin-loop structure, it has been named hairpin catalytic RNA (22). Hairpin catalytic RNA has a single-stranded region that binds substrate RNA by Watson–Crick base pairing, and kinetic analyses have shown that the catalytic center of the original (−)sTRSV sequence is the most efficient *in vitro* among the ribozymes described to date (18).

In this investigation, we tested whether or not hairpin catalytic RNA cleaves other RNAs when the sequence of the substrate binding site is altered. Synthetically constructed hairpin catalytic ribozymes containing new substrate binding sites targeted to *Escherichia coli* prolipoprotein signal peptidase mRNA (23) efficiently cleaved this mRNA *in vitro*. These ribozymes have the potential to cleave any RNA molecule containing GUC or GUA.

MATERIALS AND METHODS

Enzymes and chemicals

T7 RNA polymerase was obtained from Toyobo (Osaka). SP6 RNA polymerase, T4 polynucleotide kinase, and T4 RNA ligase were from Takara Shuzo (Kyoto). Restriction endonucleases were purchased from Toyobo, Takara Shuzo, or New England Biolab. RNase-free DNase and the plasmid pGEM-3Z were from Promega. Other enzymes and Chemicals were purchased from commercial sources.

Oligodeoxynucleotides

Oligodeoxynucleotides were synthesized using an Applied Biosystems DNA synthesizer. The sequences of the oligomers are shown in Table 1. The 5′-phosphorylated double-stranded oligonucleotides, ALSP1–3, were obtained by phosphorylation of each single-stranded oligodeoxynucleotide with ATP and T4 polynucleotide kinase and annealing.

Preparation of substrates

An *E. coli* essential gene, prolipoprotein signal peptidase (*lsp*), was used to provide RNA transcripts as a target for cleavage by newly designed ribozymes. The *lsp* gene was obtained from plasmid pMT521 (Figure 1) kindly supplied by M. Tokunaga (24). To obtain transcripts of *lsp*, the *Rsa* I-*Rsa* I fragment (bases

274 to 1068 in Figure 1) from pMT521 was cloned into the *Sma* I site of pGEM-3Z to give pYOK2 (Figure 1). The *Sau* 3AI-*Sau* 3AI fragments (bases 447 to 501 and 522 to 677) of the *lsp* gene were also cloned into the *Bam* HI sites of pGEM-3Z vectors to give pYOKS403 and pYOKS501, respectively (Figure 1). These plasmids, pYOK2, pYOKS403, and pYOKS501, were then digested with *Bss* HII, *Xba* I, and *Eco* RI, respectively. The substrates, YOK2 (532 bases), YOKS403 (88 bases), and YOKS501 (220 bases) were prepared by transcription of these linearized plasmids with T7 or SP6 RNA polymerase (Figure 1).

The reaction mixture for transcription contained 40 mM Tris-HCl (pH 7.6), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 40 units of RNasin, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, and 0.1 mM [α-^{32}P]UTP (total 10–50 μCi), 0.5 μg plasmid template, and 100 units of T7 RNA polymerase or 30 units of SP6 RNA polymerase in a total volume of 50 μl. The mixture was incubated for 1 h at 37°C, and the transcripts were precipitated in ethanol, dried, and dissolved in a urea/dye loading mixture. Then they were electrophoresed in 5 or 10% polyacrylamide/ 8 M urea gels. RNAs were located by autoradiography, eluted from the gels, precipitated with ethanol, and dried.

Preparation of ribozymes

Transcription mixtures for ribozymes were the same as described above for preparation of substrates except that either *Eco* RI-linearized pNS80, pNS82, or pNS83 (see Results) was used as the DNA template and 0.5 mM non-radioactive UTP was used instead of [α-^{32}P]UTP. Each mixture was incubated for 1 h at 37°C after which 1 μl (1 unit) of RNase-free DNase was added. Incubation then was continued for 10 min at 37°C. Following phenol extraction and ethanol precipitation, RNA was collected by centrifugation, dried, and dissolved in water at a concentration of 1 μg/μl.

Cleavage reactions

The mixture for cleavage reactions contained 40 mM Tris-HCl (pH 7.6), 12 mM $MgCl_2$, 2 mM spermidine, 30–80 fmol of ^{32}P-labeled substrate RNA, and 1 μg of ribozyme RNA in a total volume of 10 μl. Mixtures were incubated for 1 h at 37°C, 42°C, or 50°C. Reaction products were denatured by heating in urea/dye loading mixture, fractionated on 5% polyacrylamide/ 8 M urea gels, and autoradiographed.

Kinetic analyses

Kinetic analyses of two ribozyme reactions were performed. Ribozymes NS80 and NS83 were labeled with [α-^{32}P]UTP to a specific activity of 1.1×10^5 cpm/pmol. The specific activities of the substrates, YOKS403 and YOKS501, were 1.4×10^5 cpm/pmol and 3.6×10^5 cpm/pmol, respectively. The reaction mixture contained 40 mM Tris-HCl (pH 7.6), 12 mM $MgCl_2$, 2 mM spermidine, 1.5 nM ribozyme (NS80 or NS83), and 3.0, 4.5, 6.0, or 12.0 nM substrate RNA (YOKS403 or YOKS501) in a total volume of 20 μl. The mixture was incubated for 30 min at 37°C, and reactions were stopped by addition of 1 μl of 0.5 M EDTA. Then, the products were separated by electrophoresis in a 10% polyacrylamide/ 8 M urea gel. Quantitative analyses of the reactions were performed by counting photo-stimulated luminescence of the product bands in a radiogram of the gel using a Bio-Image Analyzer BAS2000 (Fuji Film Co.; ref. 25).

Cloning, nucleic acid manipulations, and analytical methods

DNA manipulations and cloning techniques were performed as described by Maniatis *et al.* (26). Labeling and other analytical methods for RNA were as described by Krupp and Gross (27).

RESULTS

Constructions of ribozymes

To obtain various types of hairpin ribozymes, we first prepared a substrate binding site exchangeable cassette using synthetic oligodeoxynucleotides TR-III and TR-IIa (Table 1). TR-IIa contained a coding sequence for the hairpin region of the catalytic RNA. Because the 3'-terminal 14 nucleotides of TR-III and TR-IIa were complementary, the two oligomers were annealed, and the long recessed 3' ends were filled in with dNTPs and *Taq* DNA polymerase as described (28). The product, substrate binding site exchangeable cassette, was purified by electrophoresis in an agarose gel. This double stranded DNA, containing a *Hin* dIII site at one end and an *Eco* RI site at the other, was digested with the two restriction enzymes. Then it was ligated into the corresponding *Hin* dIII and *Eco* RI sites of the plasmid vector pGEM-3Z to give pNON2 (Figure 2A). This cassette was designed to have an *Hpa* I recognition site at the 5' edge of the hairpin region (Figure 2A) in order to construct new plasmids with different sequences at the substrate binding site of hairpin catalytic RNA by replacing the *Hin* dIII-*Hpa* I fragment with various synthetic fragments.

Three plasmids containing ribozyme sequences each targeted to a different region of *E. coli* prolipoprotein signal peptidase (*lsp*) mRNA were constructed (23). Since natural substrates in plant viral satellite RNAs for hairpin catalytic RNAs contain GUC (3) or GUA (19, 29) sequence, three such sites were arbitrarily chosen on *lsp* mRNA. The *Hin* dIII-*Hpa* I fragment of pNON2 (Figure 2A) was replaced by synthetic double-stranded oligodeoxynucleotides ALSP1, 2, and 3 (Table 1) to give pNS80,

Table 1. Synthetic oligodeoxynucleotides used in this study.

oligo name	sequence
TR-III	5'TCAAGCTTACGCCGCGCAGACGTTAACCAGAGAAA3'
TR-IIa	5'GCGAATTCGAGTACCAGGTAATATACCACAACGTGTGTTTCTCTGGTTAAC3'
ALSP1	5'pAGCTTCCCAGATCGATAATCAGCACAGAAACC3'
	3'AGGGTCTAGCTATTAGTCGTGTCTTTGGp5'
ALSP2	5'pAGCTTAAGCGACGGGAACAGCGGAGAAGTA3'
	3'ATTCGCTGCCCTTGTCGCCTCTTCATp5'
ALSP3	5'pAGCTTCACGCTAATACCAATCGCAACGAAGGC3'
	3'AGTGCGATTATGGTTAGCGTTGCTTCCGp5'

pNS82, and pNS83, respectively. *In vitro* transcripts from the plasmids, restricted by *Eco* RI, are shown in Figure 2B. The three ribozymes, NS80, NS82, and NS83 were designed to cleave *lsp* mRNA at separate sites between nucleotides A486 and G487, G552 and G553, and G655 and G656, respectively (Figure 2B).

Cleavage activity of synthesized ribozymes

The labeled transcripts, YOK2 (532 bases), YOKS403 (88 bases), and YOKS501 (220 bases) were incubated with synthetic ribozymes NS80, NS82, or NS83, and the products were analyzed by 5% polyacrylamide gel electrophoresis (Figure 3). All three ribozymes cleaved the substrates, and two (NS80 and NS83) cleaved them efficiently. From the mobilities of products, it seemed likely that specific cleavage occurred at the predicted sites (Figure 3). However, ribozyme NS82 did not cleave these substrates efficiently (Figure 3A, lanes c and f; Figure 3B, lane k), even though a large excess of ribozyme (100:1 in molar ratio) was used. From the intensity of the product bands, no difference was apparent between the cleavage reactions at 42°C and 50°C (Figure 3A).

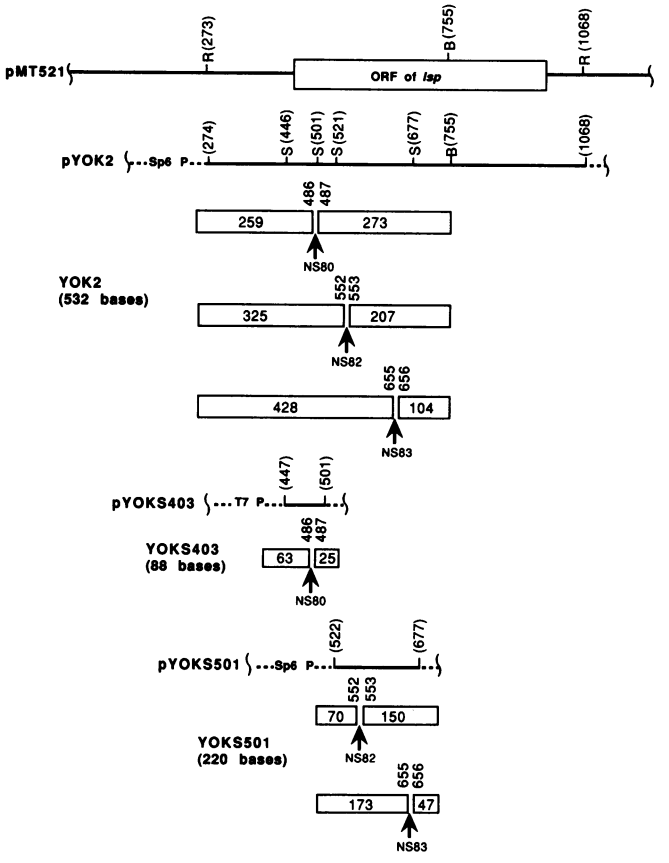

Figure 1. Construction of plasmids containing the *lsp* mRNA sequence and the location of ribozyme target sites in the transcripts. Top line indicates the *lsp* gene in plasmid pMT521 (23). Open reading frame (ORF) of *lsp* is boxed. The following restriction enzyme recognition sites are shown: R, *Rsa* I; B, *Bss* HII; S, *Sau* 3AI. Numbers indicate the nucleotide numbers of the *lsp* gene according to the sequence from Innis *et al.* (23). The *Rsa* I fragment (274–1068) and the *Sau* 3AI fragments (447–501 and 522–677) were subcloned into the pGEM-3Z vector to give pYOK2, pYOKS403 and pYOKS501, respectively. Boxes under the plasmids indicate the transcripts (YOK2, YOKS403 and YOKS501) used for ribozyme reactions as substrates. Target sites for ribozymes (NS80, NS82 and NS83) are shown by arrows. Numbers in boxes indicate expected chain lengths (bases) of cleavage products by ribozymes. Sp6 P and T7 P, promoter sequences for SP6 and T7 RNA polymerases, respectively.

Determination of ribozyme cleavage sites

To identify the cleavage sites, the terminal sequences of the cleavage products were directly determined. The 3′ fragment (47 bases) from cleavage of non-radioactive YOKS501 by ribozyme NS83 was purified by polyacrylamide gel electrophoresis. The sample could be labeled at the 5′ end using T4 polynucleotide kinase and [γ-^{32}P]ATP without prior treatment with calf intestinal alkaline phosphatase. This indicated that the 5′ end of the 3′ fragment had a free hydroxyl group. The labeled fragment was then partially digested by mild alkali treatment, and the resulting fragments were separated by two dimensional electrophoresis/ homochromatography (data not shown). The mobility shift from fragment to fragment permitted reading of the sequence 5′GUAUU3′ corresponding to bases G656 to U660 in *lsp* mRNA (see Figure 2B).

The 5′ fragment (173 bases) cleaved from the non-radioactive YOKS501 substrate by ribozyme NS83 was also purified and analyzed. This fragment could not be labeled at its 3′ end using

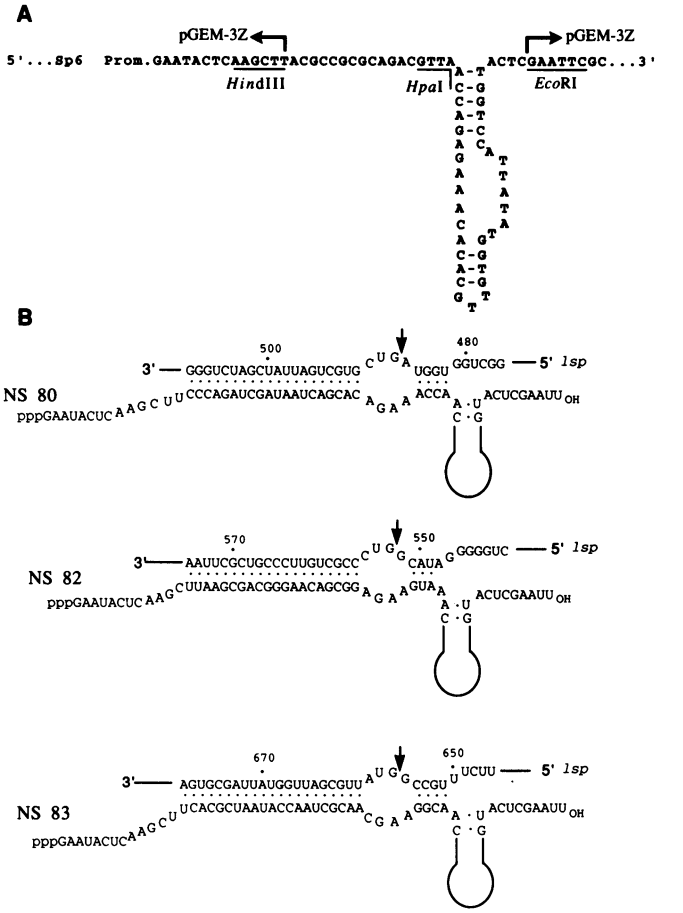

Figure 2. Nucleotide sequence of pNON2 (A) and design of ribozymes targeting the *lsp* gene transcript (B). A, Non-coding sequence of the hairpin region of pNON2 is shown. The secondary structure of the hairpin region is depicted. Sp6 Prom., promoter for SP6 RNA polymerase; B, Ribozymes termed NS80, NS82, and NS83, are directed against three sites within the *lsp* gene transcript. Arrows indicate predicted sites for RNA cleavage. Numbers are nucleotide numbers of the *lsp* gene as in Figure 1. Hairpin regions are depicted by lines.

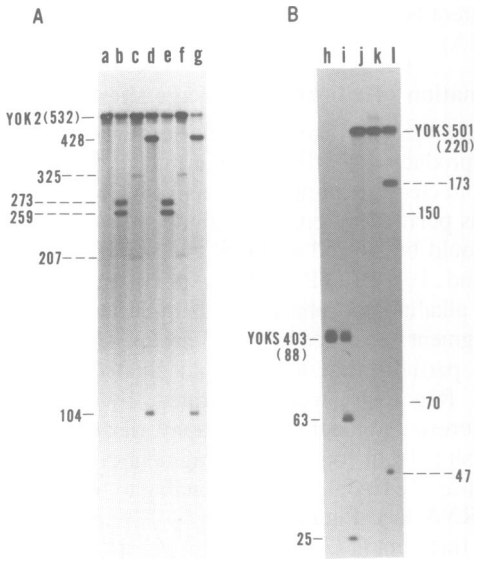

Figure 3. Cleavage of *lsp* mRNA sequences by designed ribozymes. Electrophoretic analysis of reactions using YOK2 (A), YOKS403, and YOKS501 (B) as substrates. Mixtures were analyzed in 5% polyacrylamide/ 8 M urea gels. Autoradiograms of the gels are shown. A, Lanes: a, no added ribozyme; b and e, plus NS80; c and f, plus NS82; d and g, plus NS83. Mixtures were incubated for 1 h at 42°C (lanes b, c and d) or 50°C (lanes a, e, f and g). B, Mixtures were incubated for 1 h at 42°C. Lanes: h and j, no added ribozyme; i, plus NS80; k, plus NS82; l, plus NS83. Numbers indicate expected chain lengths (bases) of the substrates and products (see Figure 1).

T4 RNA ligase and [5′-^{32}P]pCp, even after treatment with calf intestinal alkaline phosphatase, suggesting that the 3′ end was neither a hydroxyl nor a phosphomonoester group. However, 3′ labeling was possible when the sample was preincubated in 10 mM HCl and then treated with phosphatase. This indicated that it terminated with a 2′,3′ cyclic phosphodiester group. The 3′ terminal nucleotide was identified as [^{32}P]Gp by RNase T2 digestion and thin-layer chromatography of the sample (data not shown).

In sum, the results showed that ribozyme NS83 cleaved *lsp* mRNA at the predicted location and generated 2′,3′ cyclic phosphodiester and 5′ hydroxyl groups at the cleavage site. Using a similar analysis, specific cleavage of substrate YOKS403 by NS80 was also confirmed at the designed site (results not shown). Although we did not extensively analyze the cleavage site of the NS82 reaction because of the limited amount of the products, the mobilities of the product fragments in 5% polyacrylamide/ 8 M urea gels (Figure 3) suggest that cleavage also occurred correctly.

Kinetics of cleavages

Kinetic parameters of two ribozyme reactions, those of NS80 and NS83, were measured using a Lineweaver-Burk plot. 1.5 nM of each ribozyme and 3.0−12.0 nM of the substrates were mixed, and the reaction was performed at 37°C. Apparent K_m and k_{cat} values are shown in Table 2. k_{cat} values of the NS83 and NS80 reactions were found to be 0.033 and 0.008 (min^{-1}), respectively. These are much smaller than that of the original (−)sTRSV sequence. One explanation may be that the long substrate binding sites (see Figure 2B) impaired dissociation of the product fragments, especially 3′ fragments, from the ribozymes after cleavage. k_{cat}/K_m values of NS83 and NS80 were calculated to be 1.32 and 0.33, respectively. Although these values are also much smaller than that of the original (−)sTRSV sequence, they are comparable to that of the well-known hammerhead ribozyme (Table 2).

Table 2. Kinetic parameters of ribozymes.

	K_m(μM)	k_{cat}(min^{-1})	k_{cat}/K_m	References
501+NS83	0.025	0.033	1.32	This work
403+NS80	0.023	0.008	0.33	This work
(−)sTRSV	0.03	2.1	70	(18)
Hammerhead	0.62	0.5	0.81	(7)

DISCUSSION

The present study demonstrates that newly designed hairpin ribozymes can cleave natural mRNA sequences of various sizes, including a transcript of 0.5 kilobases. Two ribozymes (NS80 and NS83) out of three efficiently cleaved substrate RNAs, even though the target sites were arbitrarily chosen. This suggests that hairpin ribozymes have the potential to cleave any RNA molecule containing a GUC or GUA sequence. By contrast, NS82 did not efficiently cleave the substrates. Possible reasons for inefficient cleavage with NS82 include the following: 1) NS82 contains some sequence(s) unfavorable for cleavage; 2) as described for hammerhead ribozymes (30), the substrate site may have a folded structure preventing ribozyme binding; 3) the three base pairs at positions 549 to 551 of the *lsp* sequence and the NS82 (see Figure 2B) may not be sufficient for efficient cleavage. In the case of NS80 and NS83, four base pairs with the substrates are possible at this location (Figure 2B). Further studies are needed to elucidate the molecular mechanism underlying this difference in cleavage efficiency.

In order to give high specificity to the ribozymes, especially taking into account *in vivo* expression, they were designed with relatively long substrate binding sites (Figure 2B). This may be the cause of the very small k_{cat} values of the reactions. However, the K_m values were also small, and the calculated k_{cat}/K_m values were comparable to that of the hammerhead ribozyme. Values for the hammerhead ribozyme were derived from a reaction at 55°C (7), so it is possible that NS80 and NS83 may be catalytically more efficient than the hammerhead ribozyme at the physiological temperature of 37°C. Recently, it has been reported that the turnover of a hammerhead ribozyme reaction increases when the number of base pairs formed with the substrate is reduced (17). In the case of our ribozymes, it is also possible that the k_{cat} value may increase after shortening the substrate binding site. The optimum length of the site will have to be determined empirically.

Expression of NS80 and NS83 also was tested *in vivo*. However, in both cases production of the prolipoprotein signal peptidase was not inhibited by the presence of ribozyme in the *E. coli* cells (Kikuchi and Sasaki, unpublished result). Transcription and translation are coupled in *E. coli*, so it is likely that NS80 and NS83 could not interact with target mRNA before its translation. Similar results have been reported for the hammerhead ribozyme in *E. coli* (31). Consequently, eucaryotic cells or viruses including bacteriophages may be better systems for studies of the role of ribozymes in the control of gene expression *in vivo*. In fact, some newly designed hammerhead ribozymes have been reported to function in eucaryotic cells

(12–14, 32). The hairpin ribozyme seems to be catalytically more efficient than the hammerhead ribozyme, at least *in vitro*. Further investigations will reveal its potential for creating more efficient ribozyme systems *in vivo*.

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